

A new blood-brain barrier model using primary rat brain endothelial cells,
pericytes and astrocytes

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Abstract

Blood-brain barrier (BBB) characteristics are induced and maintained by cross-talk between brain microvessel endothelial cells and neighbouring elements of the neurovascular unit. While pericytes are the cells situated closest to brain endothelial cells morphologically and share a common basement membrane, they have not been used in co-culture BBB models for testing drug permeability. We have developed and characterized a new syngeneic BBB model using primary cultures of the three main cell types of cerebral microvessels. The co-culture of endothelial cells, pericytes and astrocytes mimick the anatomical situation *in vivo*. In the presence of both pericytes and astrocytes rat brain endothelial cells expressed enhanced levels of tight junction (TJ) proteins occludin, claudin-5 and ZO-1 with a typical localization at the cell borders. Further morphological evidence of the presence of interendothelial TJs was provided by electron microscopy. The transendothelial electrical resistance (TEER) of brain endothelial monolayers in triple co-culture, indicating the tightness of TJs reached $400 \Omega \times \text{cm}^2$ on average, while the endothelial permeability coefficients (P_e) for fluorescein was in the range of $3 \times 10^{-6} \text{ cm/s}$. Brain endothelial cells in the new model expressed glucose transporter-1, efflux transporters P-glycoprotein and multidrug resistance protein-1, and showed a polarized transport of rhodamine 123, a ligand for P-glycoprotein. To further characterize the model, drug permeability assays were performed using a set of 19 compounds with known *in vivo* BBB permeability. Good correlation ($R^2=0.89$) was found between *in vitro* P_e values obtained from measurements on the BBB model and *in vivo* BBB permeability data. The new BBB model, which is the first model to incorporate pericytes in a triple co-culture setting, can be a useful tool for research on BBB physiology and pathology and to test candidate compounds for centrally acting drugs.

Keywords: Blood-brain barrier; Brain endothelial cells; Pericytes; Astrocytes; Co-culture; *In vitro* BBB model (rat); Tight junction; P-glycoprotein; Transendothelial electrical resistance; Drug permeability

Running title: Blood-brain barrier model with pericytes

1. Introduction

Cell-culture based models have greatly contributed to our knowledge on the physiology, pathology and pharmacology of the blood-brain barrier (BBB) (Deli et al., 2005; Cecchelli et al., 2007). Since 1973, when the first *in vitro* model of the BBB was established by the successful isolation of brain microvessels (Joó and Karnushina, 1973) several models constructed from cultured cells have been developed (Deli, 2007). The mono-cultures of brain endothelial cells were replaced by co-culture systems when it was recognized that (i) cerebral endothelial cells lose easily their specific characteristics in culture and (ii) the cells of the neurovascular unit play an important role in the induction of BBB properties (Abbott et al., 2006; Cecchelli et al., 2007). The BBB phenotype of brain endothelial cells includes tight junctions (TJs) and the lack of pinocytosis and fenestrae restricting passage of solutes and cells to the brain, transendothelial transport pathways, metabolic and detoxifying functions (Abbott et al., 2006; Neuwelt et al., 2008). By these specialized functions the BBB provides ionic homeostasis and nutrients necessary for the proper functioning of the CNS and it protects the nervous system from xenobiotics and regulates the level of neuroactive mediators (Pardridge, 2002; Abbott et al., 2006; Zlokovic 2008).

Brain capillary endothelial cells have a dynamic interaction with other neighbouring cells, astroglia, pericytes, perivascular microglia and neurons. This cooperation contributes to their unique characteristics displaying both endothelial and epithelial features (Joó, 1996; Deli et al., 2005; Abbott et al., 2006; Cecchelli et al., 2007). The cross-talk between the cells of the neurovascular unit is crucial for the formation and maintenance of a functional BBB (Abbott et al., 2006; Zlokovic, 2008). Among these cells, astrocytes were the first to be recognized as regulators of brain endothelial characteristics and functions (for reviews see Abbott, 2005; Haseloff et al., 2005; Abbott et al., 2006). Astrocytes are able to induce among others the formation of interendothelial tight junctions (TJs) (Tao-Cheng et al., 1987), a fundamental characteristic of the BBB. A great number of currently used *in vitro* BBB model is composed of co-culture of brain endothelial cells with astrocytes (Deli et al., 2005; Deli 2007; Cecchelli et al., 2007).

Brain pericytes the nearest neighbours of endothelial cells sharing a common basal membrane in cerebral capillaries received much less attention so far (Lai and Kuo, 2005). Despite their localization and fundamental role in stabilizing brain capillary structure *in vivo* (Hellstrom et al., 2001; Lai and Kuo, 2005), and their possible importance in the development, maintenance, and regulation of the BBB (Lai and Kuo, 2005; Zlokovic, 2008) few data are

available on the functional significance of pericytes on BBB properties. Pericytes were found to be able to tighten the paracellular barrier in cultured brain endothelial cells (Hayashi et al., 2004; Dohgu et al., 2005) similarly to astrocytes. We have recently performed a systematic comparison of 7 different types of BBB models constructed from primary cultures of rat brain microvessel endothelial cells, pericytes and astrocytes (Nakagawa et al., 2007). Transendothelial electrical resistance (TEER) and permeability for a small water-soluble tracer fluorescein were measured to evaluate paracellular transport reflecting TJ function, an important parameter of the quality of BBB models (Deli et al., 2005). We confirmed that brain pericytes could strengthen the barrier integrity of cerebral endothelial monolayers. It was demonstrated that a triple co-culture model consisting of brain endothelial cells and pericytes grown on the opposite sides of a porous membrane and cultured in the presence of astrocytes was superior in barrier integrity to the other BBB models tested (Nakagawa et al., 2007). This *in vitro* BBB model corresponds to the anatomical situation in the cerebral microvessels.

The aim of the present study was the detailed characterization of the new triple co-culture BBB model. We examined the cytoarchitecture and cellular markers by immunofluorescence and electron microscopy, the morphological and functional integrity of the paracellular barrier and the presence and function of influx and efflux transporters. Finally *in vitro* drug permeability was tested for 19 compounds on the triple co-culture BBB model and compared to *in vivo* permeability data in the same species.

2. Materials and Methods

All reagents used in the study were purchased from Sigma (St. Louis, MO, USA), unless otherwise indicated. Wistar rats were obtained from Japan SLC Inc., (Shizuoka, Japan). Balb/c mice were obtained from Charles River Laboratories Japan Inc., (Kanagawa, Japan). All animals were treated in strict accordance with the NIH Guide for Care and Use of Laboratory Animals (NIH Publications No. 80-23) and as approved by the Nagasaki University Animal Care Committee.

2.1. Cell cultures

Primary cultures of rat brain capillary endothelial cells (RBEC) were prepared from 3-week-old rats, as previously described (Deli et al., 1997). Meninges were carefully removed

from forebrains and gray matter was minced into small pieces of approximately 1 mm³ in ice-cold Dulbecco's modified Eagle's medium (DMEM), then dissociated by 25-times of up-and-down-strokes with a 5-ml pipette in DMEM containing collagenase type 2 (1 mg/ml, Worthington, USA), 300 µl DNase (15 µg/ml), gentamycin (50 µg/ml) and then digested in a shaker for 1.5 h at 37 °C. The cell pellet was separated by centrifugation in 20 % bovine serum albumin (BSA)-DMEM (1000 × g, 20 min). The microvessels obtained in the pellet were further digested with collagenase-dispase (1 mg/ml, Roche, Switzerland) and DNase (6.7 µg/ml) in DMEM for 1 h at 37 °C. Microvessel endothelial cell clusters were separated on a 33 % continuous Percoll (Pharmacia, Sweden) gradient, collected and washed twice in DMEM before plating on 35 mm plastic dishes coated with collagen type IV and fibronectin (both 0.1 mg/ml). RBEC cultures were maintained in DMEM/F12 supplemented with 10 % plasma derived serum (PDS, Animal Technologies, USA), basic fibroblast growth factor (bFGF, Roche, Switzerland, 1.5 ng/mL), heparin (100 µg/ml), insulin (5 µg/ml), transferrin (5 µg/ml), sodium selenite (5 ng/ml) (insulin-transferrin-sodium selenite media supplement), gentamycin (50 µg/ml) and puromycin (4 µg/ml) (Perrière et al., 2005) (RBEC medium I) at 37 °C with a humidified atmosphere of 5% CO₂/95 % air, for 2 days. On the third day, the cells received a new medium which contained all the components of RBEC medium I except puromycin (RBEC medium II). When the cultures reached 80% confluency (4th day *in vitro*), the purified endothelial cells were passaged by a brief treatment with trypsin (0.05 % wt/vol)-EDTA (0.02 % wt/vol) solution, and used to construct various types of *in vitro* BBB models (Figs. 1 and 4).

Rat cerebral astrocytes were obtained from neonatal Wistar rats. Meninges were removed and cortical pieces mechanically dissociated in astrocyte culture medium (DMEM supplemented with 10 % fetal bovine serum). Dissociated cells were seeded into cell culture flasks. In order to obtain type 1 astrocytes, flasks with confluent cultures were shaken at 37 °C overnight. The purity of astrocytes was checked by immunostaining for glial fibrillary acidic protein (GFAP), and the cells were used at passage 2.

Pure cultures of rat cerebral pericytes were obtained by a prolonged, 2-week culture of isolated brain microvessel fragments, that contain pericytes beside endothelial cells. The same preparations yield primary RBEC after puromycin-treatment. Pericyte survival and proliferation was favored by selective culture conditions, using uncoated dishes, and DMEM supplemented with 10 % fetal bovine serum and antibiotics. Culture medium was changed every 3 days. Pericytes were characterized by their large size and branched morphology, positive immunostaining for α -smooth muscle actin, NG2 chondroitin sulfate proteoglycan

and absence of von Willebrand factor and glial fibrillary acidic protein (GFAP) staining. Pericytes and astrocytes were frozen in cryo-medium Cellbanker (BCL-1, Zenoaq, Koriyama, Japan), and stored at -80 °C until use.

2.2. Construction of *in vitro* BBB models

The day when the endothelial cells were plated and models were established was defined as day zero *in vitro* (Day 0; Fig. 1). To construct various *in vitro* models of BBB pericytes or astrocytes (1.5×10^4 cells/cm²) were seeded on the bottom side of the collagen-coated polyester membrane of the Transwell inserts. The cells were let to adhere firmly for overnight, then endothelial cells (1.5×10^5 cells/cm²) were seeded on the inside or upper side of the inserts placed in the well of the 12-well culture plates containing no cells, pericytes or astrocytes (Fig. 4). From Day 1 BBB models were maintained in RBEC medium II supplemented with 500 nM hydrocortisone (Hoheisel et al., 1998). Under these conditions, *in vitro* BBB models were established within 3 days after setting of the cells. Seven types of BBB models were constructed (Fig. 4; Nakagawa et al., 2007). As negative controls for barrier integrity studies, astrocytes and pericytes, which do not form barrier, were cultured on the inserts, respectively.

2.3. Immunostaining

To characterize the cultures after washing and fixation brain endothelial cells were incubated with anti-von Willebrand factor rabbit polyclonal antibody, astrocytes with anti-GFAP mouse monoclonal antibody (Progen Scientific Ltd., Mexborough, UK), pericytes with anti-NG2 chondroitin sulfate proteoglycan rabbit polyclonal antibody (Millipore Corp., MA, USA). All primary antibodies were used in a dilution 1:100. As secondary antibodies Alexa Fluor 488 conjugated donkey anti-rabbit and anti-mouse immunoglobulins (both from Invitrogen Corporation, CA, USA) were used in a dilution 1:1000. To counterstain cell nuclei TO-PRO-3 Iodide (Invitrogen Corporation, CA, USA) was used in a dilution of 1:400. To stain brain endothelial junctional proteins cell monolayers cultured on inserts in the absence or presence of glial cells and pericytes were stained for ZO-1 and claudin-5. The cultures were washed in PBS and fixed with ethanol (95 v%)-acetic acid (5 v%) for 10 min at - 20 °C (ZO-1) or with ethanol for 30 min at 4 °C (claudin-5). Cells were blocked with 3 % BSA and incubated with primary antibodies anti-ZO-1 and anti-claudin-5 (Zymed Laboratories Inc.,

CA, USA), for 1 h 30 min. Incubation with secondary antibody Cy3-labelled anti-rabbit IgG lasted for 1 h. Between incubations cells were washed three times with PBS. Preparations were mounted in Gel Mount (Biomedica, USA) and staining was examined by a Zeiss LSM 5 Pascal Confocal laser scanning microscope (Oberkochen, Germany).

2.4. Electron microscopy

Cells grown on the membrane were fixed with 3 % paraformaldehyde in cacodylate buffer (pH 7.5) for 30 min at 4 ° C. After washing with cacodylate buffer several times, the membranes of the culture inserts with the cells on the two sides were removed from their support and placed into 24 -well chamber slide and were postfixed in 1 % OsO₄ for 30 min. Following washing with distilled water, the cells on the membrane were dehydrated in graded ethanol, block-stained with 2 % uranyl acetate in 70 % ethanol for 1 h and embedded in Taab 812 (Taab; Aldermaston, Berks, UK). Ultrathin sections were cut perpendicularly for the membrane using a Leica UCT ultramicrotome (Leica Microsystems, Milton Keynes, UK) and examined using a Hitachi 7100 transmission electron microscope (Hitachi Ltd., Tokyo, Japan).

2.5. Evaluation of the barrier integrity

TEER reflecting the flux of mainly sodium ions through cell layers in culture conditions was measured by Epithelial-volt-ohm meter and Endohm-12 chamber electrodes (World Precision Instruments, USA). TEER of coated, but cell-free filters was subtracted from measured TEER values of the models shown as $\Omega \times \text{cm}^2$. The flux of sodium fluorescein (Na-F) across endothelial monolayers was determined as previously described (Kis et al., 2001; Veszelka et al., 2007). Cell culture inserts were transferred to 12-well plates containing 1.5 ml Ringer-Hepes buffer (136 mM NaCl, 0.9 mM CaCl₂, 0.5 mM MgCl₂, 2.7 mM KCl, 1.5 mM KH₂PO₄, 10 mM NaH₂PO₄, 25 mM glucose and 10 mM Hepes, pH 7.4) in the lower or abluminal compartments. In the inserts (luminal compartment) culture medium was replaced by 0.5 ml buffer containing 10 $\mu\text{g/ml}$ Na-F (MW: 376 Da). The inserts were transferred at 5, 15 and 30 min to a new well containing Ringer-Hepes buffer. The concentrations of the marker molecule in samples from the upper and lower compartments were determined by fluorescence multiwell plate reader Wallac 1420 ARVO Multilabel Counter (Perkin Elmer, USA; excitation: 485 nm, emission: 535 nm). Flux across cell-free inserts was also measured.

Transendothelial permeability coefficient (P_e) was calculated as previously described (Deli et al., 2005; Veszelka et al., 2007) and as detailed in data analysis.

2.6. Western blotting

Protein samples from brain endothelial cells cultured in the presence or absence of pericytes and astrocytes were separated by SDS-PAGE and transferred to nitrocellulose (Hybond, GE Healthcare, UK). Non-specific binding sites were blocked by Perfect-Block (MoBiTec GmbH, Germany) (1 % w/v) in Tris-buffered saline (TBS; 25 mM Tris, 150 mM NaCl, 2 mM KCl, pH 7.4) containing 0.1 % Tween-20. Anti-claudin-5 and anti-occludin mouse monoclonal antibodies, anti-ZO-1 rabbit polyclonal antibody (all from Zymed Laboratories Inc., CA, USA), anti-Mdr rabbit and anti-MRP1 goat polyclonal antibodies (Santa Cruz Biotechnology, Inc., CA, USA), anti-glucose transporter-1 rabbit polyclonal antibody (Millipore Corp., MA, USA) were used in a dilution of 1:5000 in blocking solution to incubate blots for 1 h at room temperature. Peroxidase-conjugated anti-mouse, anti-rabbit and anti-goat immunoglobulins (GE Healthcare, UK) were applied as secondary antibodies. Between incubations blots were washed three times with TBS. To reveal immunoreactive bands the blots were incubated in ECL Plus reagent following the manufacturers instructions (GE Healthcare, UK) and detected by FluorChem SP Imaging System (Alpha Innotech Corp., CA, USA).

2.7. Functional assay for P-glycoprotein

Activity of P-glycoprotein was determined by the measurement of the polarity of the transport of rhodamine 123, a ligand of P-glycoprotein (Fontaine et al., 1996). In brief, the inserts containing the cell layers were gently washed and the flux of 1 μ M rhodamine 123 in Ringer-Hepes buffer was measured for 1 h at 37°C in the luminal-to-abluminal and in the opposite abluminal-to-luminal directions. Rhodamine 123 content in both compartments was determined by Wallac 1420 ARVO Multilabel Counter (Perkin Elmer, USA; excitation at 485, emission at 538 nm). Verapamil (2 μ M, 30 min preincubation) was used as a reference P-glycoprotein inhibitor.

2.8. In vitro drug transport experiments

To measure the flux of 19 drugs selected for the study (Table 1) across the endothelial and pericyte monolayers cell culture inserts following measurement of TEER were transferred to 12-well plates containing 1.5 ml Ringer-Hepes solution in the lower compartments. All test compounds were dissolved in DMSO to yield a 1 mM solution, which was further diluted in Ringer-Hepes buffer. In the upper or luminal chambers culture medium was replaced by 0.5 ml Ringer Hepes containing the test compounds at 1 μ M concentration. The inserts were transferred at 20, 40 and 60 min to a new well containing Ringer-Hepes solution. The concentrations of the test molecules in samples from the upper (luminal) and lower (abluminal) compartments were determined by high performance liquid chromatography. P_e was calculated for each drug as described in data analysis.

2.9. *In vivo studies of drug permeability in mice*

The distribution of compounds to brain tissue *in vivo* was measured using a tissue distribution model in mice (Garberg et al., 2005). Five min after the injection of a single dose of the compounds in anesthetized mice via tail vein (n=3), blood samples were collected from vena cava and the whole brain was removed. The concentration of the compounds in brain and plasma samples were measured by liquid chromatography-tandem mass spectrometry (LC-MS/MS). The ratio of the concentration in brain and plasma (K_p) was determined for each drug. The ratio was then used to calculate the apparent permeability coefficient (P_{app}), as described in section 2.10., presuming that metabolism, back-flux and tissue accumulation are negligible at that time point.

2.10. *Analysis of permeability data and correlation with in vivo results*

Transport was expressed as μ l of donor (luminal) compartment volume from which the tracer is completely cleared. Transendothelial permeability coefficient (P_e) was calculated as previously described (Deli et al., 2005; Veszeka et al., 2007). Cleared volume was calculated from the concentration (C) of the tracer in the abluminal and luminal compartments and the volume (V) of the abluminal compartment (0.5 ml) by the following equation:

$$\text{Cleared volume } (\mu\text{l}) = \frac{C_{abluminal} \times V_{abluminal}}{C_{luminal}}$$

The average cleared volume was plotted vs. time, and permeability \times surface area product value for endothelial monolayer (PS_e) was calculated by the following formula:

$$\frac{1}{PS_{endothelial}} = \frac{1}{PS_{total}} - \frac{1}{PS_{insert}}$$

PS_e divided by the surface area (1 cm² for Transwell-12) generated the endothelial permeability coefficient (P_e ; in 10⁻⁶ cm/s).

The *in vivo* data were obtained as described in section 2.9. Brain uptake assays were performed with the compounds, and *in vivo* P_{app} values were calculated from the ratio between the concentration in the plasma and brain tissue at 5 min and expressed as cm/s (Ohno et al., 1978, Garberg et al., 2005). The capillary surface area value of 240 cm²/g brain was used for the calculations (Garberg et al., 2005).

2.11. Statistical analysis

All data presented are means ± S.E.M. The values were compared using the analysis of variance followed by Bonferroni-Dunn test. Changes were considered statistically significant at $P < 0.05$. All experiments were repeated at least three times, and the number of parallel inserts was four.

3. Results

3.1. Characterization of the cells of the triple co-culture BBB model by immunofluorescence and electron microscopy

Rat brain endothelial cells obtained by the puromycin purification method (Fig. 1; Perrière et al., 2005; Veszelka et al., 2007; Nakagawa et al., 2007) grow in non-overlapping continuous monolayers and show tightly apposed, elongated, fusiform morphology and positive immunostaining for *von* Willebrand factor, a marker for endothelium (Fig. 2). Astroglia cells characterized by GFAP immunostaining are polygonal with long cell processes resembling astrocytic endfeet and indicating a differentiated phenotype could be also observed (Fig. 2). The shape and size of brain microvascular pericytes are very different from the other two cell types of the neurovascular unit (Fig. 2). Pericytes in culture spread large with irregular projections. They may grow in multiple layers and are positive for pericyte-markers NG2 chondroitin sulfate proteoglycan, nestin (data not shown) and α -smooth muscle actin and negative for *von* Willebrand factor and GFAP staining (Fig. 2). When brain endothelial cells were cultured in the presence of both pericytes and astrocytes (EPA model Fig. 1) smooth luminal membrane, long and oval cell nuclei (Fig. 3A), a great number of mitochondria (Fig.

3B), and interendothelial tight junctions (Fig. 3C) could be observed by electron microscopy. The cell nuclei of brain pericytes were more round and the cell bodies protruding (Fig. 3D). No junctions were present between pericytes (Fig. 3D). Long thin pericytic cytoplasmic processes were also typical (Fig. 3E).

3.2. Paracellular permeability of the BBB models

As compared to brain endothelial cell monolayers which received no influence from other cells (E00 on Fig. 4), all co-culture models showed higher TEER and lower permeability for fluorescein (Fig. 4). Astrocytes (EA0 and E0A) could increase the tightness of endothelial monolayers more than two-fold. The effect was higher when astrocytes were positioned on the bottom-side of the inserts in the in contact type model EA0. The presence of pericytes (EP0 and E0P) elevated the TEER of RBEC by four-fold on culture day 4 (Fig. 4), an effect that was significantly higher than that of astrocytes standardly used in BBB models. Triple co-culture models (EAP and EPA) were thighter than the double co-culture models as reflected by the TEER values. The resistance of EPA model increased up to $354 \pm 15 \Omega \times \text{cm}^2$ at day 4, the highest value of the seven models tested which significantly differs from all other models (Fig. 4). High TEER was observed in the models constructed with pericytes, as compared with those without pericytes. In addition, endothelial cells in contact with pericytes (EP0, EPA) showed higher TEER than those out of contact with pericytes (E0P, EAP). Astrocytes or pericytes do not form any barrier, as reflected by their very low resistance which never exceeded $10 \Omega \times \text{cm}^2$. The paracellular permeability of RBEC monolayers measured by the water soluble small marker fluorescein was the highest in endothelial monocultures (E00; $6.6 \pm 0.1 \times 10^{-6} \text{ cm/s}$), indicating the leakiest barrier (Fig. 4). The presence of astrocytes or pericytes in either double or triple co-culture systems significantly decreased the flux of the tracer. A low permeability for Na-F ($3.9 \pm 0.2 \times 10^{-6} \text{ cm/s}$) could be measured on EPA model of the BBB (Fig. 4).

3.3. Expression and localization of interendothelial tight junction proteins in the BBB models

The levels of TJ proteins occludin, claudin-5 and ZO-1 in brain endothelial cells cultured in the absence or presence of astrocytes and pericytes were determined by Western-blot (Fig. 5). The presence of astrocytes and pericytes increased the expression of endothelial TJ proteins. The highest relative levels of claudin-5 and ZO-1 were measured in the triple co-

culture model EPA, the anatomically correct model, and differed significantly from endothelial mono-cultures (E00, Fig. 5). Similar tendency could be observed for occludin.

Examination of brain endothelial monolayers by immunohistochemistry revealed that both E00 and EPA models show strong staining for intercellular junction proteins ZO-1 and claudin-5 at cell-cell contacts (Fig. 6). In endothelial cells grown in the absence of any cellular interaction (E00) irregular, „zipper-like” localization of the TJ proteins could be observed. Punctate distribution in the cytoplasm close to the cell junctions was also visible (Fig. 6 arrows). In contrast localization of ZO-1 and claudin-5 was more restricted to intercellular junctions delineating clearly cell borders in brain endothelial cells grown in the triple co-culture (EPA model; Fig. 6 arrowheads). Zipper-like irregularities and cytoplasmic localizations were also reduced in these cells.

3.4. Expression of transporters in the triple co-culture model

Brain endothelial cells in the EPA model express the BBB-specific transporter for hexoses, glucose transporter-1, revealed by western blotting as a major band at 45 kDa and a weaker band of 55 kDa isoform, both described as glucose transporter-1 isoforms in brain (Fig. 7; Yu and Ding, 1998). A strong and even distribution of glucose transporter-1 was observed in brain endothelial cells by immunohistochemistry. Among the efflux pumps present at the BBB Mrp1 was detected as a single immunoreactive band of 190 kDa (Ferneti et al., 2001) and P-glycoprotein as a band at 170 kDa (Rao et al., 2005). Both pumps could be also visualized in brain endothelial cells by immunohistochemistry (Fig. 7). In addition, the functional activity of Pgp was also tested on the EPA model using rhodamine 123 as a ligand. The abluminal to luminal (brain-to-blood) transport of the Pgp ligand was 2.5 times higher than in the opposite direction, indicating a strong efflux.

3.5. Drug permeability and correlation with in vivo data

The triple co-culture EPA model was further characterized by testing drug permeability. For these assays 19 compounds with known permeability properties including passive and active transport at the level of BBB were selected (Table 1). All molecules which enter the nervous system by lipid-mediated free diffusion and exert a central effect, antipyrin, caffeine, carbamazepine, hydroxyzine, phenytoin, propranolol, trazodone and zolpidem (indicated as CNS+ drugs) displayed a high P_e ($147.36 - 1174.04 \times 10^{-6}$ cm/s) when measured

on the model (Fig. 8). In contrast, the P_e value of hydrophilic small molecules with passive diffusion, like atenolol, or that of efflux pump ligands, like cimetidine, digoxin, epinastine, hydrocortisone, hydroxyzine, prazosin, quinidine, sulpiride, verapamil, vinblastine, vincristine, that all have low BBB permeability *in vivo* (indicated as CNS- drugs) was also small when tested on the *in vitro* BBB model ($0.44 - 23.51 \times 10^{-6}$ cm/s; Fig. 8). When the data obtained on the *in vitro* model were compared with *in vivo* data expressed as P_{app} for the same compounds a correlation of $R^2 = 0.89$ was found (Fig. 9).

4. Discussion

In the present study we have constructed and characterized a novel BBB model consisting of the triple co-culture of primary rat brain endothelial cells, pericytes and astrocytes with the aim to produce a tool for research on BBB physiology, pathology and pharmacology.

4.1. Development of the *in vitro* BBB model: methodical considerations

To establish the model three advances in culture techniques have been exploited as shown on Fig. 1. (i) The puromycin method (Perrière et al., 2005 and 2007; Calabria et al., 2006; Veszelka et al., 2007; Nakagawa et al., 2007) was used to obtain pure cultures of rat brain endothelial cells on which all further experiments were based. The principle of the method is that endothelial cells of cerebral capillaries express much higher amounts of efflux pumps especially P-glycoprotein than any other cells in the freshly isolated brain microvessel fractions and tolerate the otherwise toxic concentrations of P-glycoprotein ligand drugs while non-endothelial cells are eliminated. Puromycin was found to be the best among the P-glycoprotein ligands to selectively kill contaminating cells during the first 2 days of the culture (Perrière et al., 2005). This selection can also favor capillary endothelial cells *versus* those from larger microvessels, and this could lead to tighter monolayers and better BBB models (Ge et al., 2005).

(ii) Glucocorticoid receptor agonists either physiological like corticosterone and hydrocortisone or pharmacological like dexamethasone are known to improve the tightness of brain endothelial cells and are used in BBB models (for reviews see Deli et al., 2005; Deli, 2007). Dexamethasone an effective synthetic glucocorticoid hormone strengthens barrier properties, increases TEER and decreases P_e for paracellular markers in many models (for

reviews see Deli et al., 2005; Deli, 2007). Physiological concentration of hydrocortisone considerably improves the barrier properties of porcine cerebral endothelial cells in serum-free culture conditions (Hoheisel et al., 1998). Corticosterone is the major glucocorticoid hormone in rodents not having hydrocortisone, however both corticosterone and hydrocortisone are effective in improving paracellular tightness in cultured rat brain endothelial cells (Calabria et al., 2006). Hydrocortisone with more potent anti-inflammatory properties was more effective enhancer of the barrier properties in rat cerebral endothelial monolayers than corticosterone (Calabria et al., 2006). These results are in agreement with previous observations that hydrocortisone improved the barrier properties in both rat (Perrière et al., 2005) and mouse BBB models (Weidenfeller et al., 2005). Further studies on rat BBB models (Perrière et al., 2007; Veszelka et al., 2007; Nakagawa et al., 2007) and our present data all confirm the pharmacological effect of hydrocortisone on rat brain endothelial cells.

(iii) Cell culture inserts enabled the establishment of BBB models using three cell types. Previous triple co-culture BBB models used combinations of brain endothelial cells or cell lines, astrocytes, neurons, and leukocytes (for review see Deli, 2007). The presence of both astrocytes and neurons decreased the paracellular permeability of RBE4 immortalized rat brain endothelial cells (Schiera et al., 2005). In a flow-based *in vitro* BBB model, the differentiation of serotonergic neurons was promoted by the endothelial-glia co-culture (Stanness et al., 1999). Although the new model characterized in this study is not the first BBB model using three cell types, this is the first rat primary culture-based syngeneic model that uses brain pericytes and corresponds to the anatomical situation in brain capillaries (Nakagawa et al., 2007).

4.2. *The role of pericytes in the induction of BBB properties*

Pericytes are crucial in the angiogenesis of the nervous system. Growing microvessels of the human telencephalon are formed by a pericyte-driven angiogenic process in which the endothelial cells are preceded and guided by migrating pericytes (Virgintino et al., 2007). Pericytes also contribute to the maturation and maintenance of BBB properties (for review see Lai and Kuo, 2005). Pericytes form direct interdigitating contacts with vascular endothelial cells *in vivo* and there is a clear correlation between higher ratio of pericytes *versus* endothelial cells in blood vessels and the tightness of the endothelial barrier (Allt and Lawrenson, 2001). The barrier tightening effect of pericytes was demonstrated on *in vitro* BBB models (Hayashi et al., 2004; Dohgu et al., 2005; Nakagawa et al., 2007). Pericytes were

found to be even more effective inducers than astrocytes of paracellular tightness in primary rat brain endothelial cells (Nakagawa et al., 2007). While several observations found that pericytes or extracellular matrix from pericytes (Hartmann et al., 2007) improve the tightness of brain endothelial cells, interestingly, pericyte-endothelial interaction increased matrix metalloproteinase-9 secretion and did not improve barrier tightness in a porcine BBB model (Zozulya et al., 2008). The differences between species, the models and the culture conditions including the preparation of pericyte cultures may explain the dissimilar results.

Pericytes used in our study were derived from the same brain microvessel fraction from which endothelial cells were obtained, but they were cultured separately in conditions favoring pericyte growth. The morphology of brain pericytes revealed by light and electron microscopy was typical for this cell type (Dore-Duffy, 2008). While smooth muscle cells can also express α -smooth muscle actin, the expression of markers NG2 chondroitin sulfate proteoglycan and nestin are indicative for brain pericytes (Dore-Duffy, 2008). These pericytes together with astrocytes in the presence of hydrocortisone induced in the puromycin-purified primary brain microvascular endothelial cells a cytoarchitecture typical for the BBB. The tightly apposed, elongated, fusiform cells expressing the endothelial marker von Willebrand factor grew in monolayers in a contact inhibited fashion and were connected by tight junction. Glucocorticoids can exert a direct action on endothelial cells by switching endothelial morphology from larger, cobblestone appearance to smaller spindle shape and increasing endothelial cell density via an anti-apoptotic effect (Calabria et al., 2006). In addition, an indirect glucocorticoid effect has been suggested through astrocytes and brain pericytes by upregulation of angiopoietin-1 and downregulation of VEGF resulting in the stabilization of barrier properties at the BBB (Kim et al., 2008).

4.3. Characterization of BBB properties in the new triple co-culture model

The paracellular permeability of the new model with TEER of 350-600 $\Omega \times \text{cm}^2$ (measured on a 1 cm^2 surface with a standard, commercially available instrument; results from more than 40 preparations and 600 filters during a period of 2 years) and fluorescein P_e between 1.8 - 4 $\times 10^{-6}$ cm/s is more restrictive than any cell line based BBB model and is among the best primary cell based BBB models (for reviews see Deli et al., 2005 and Deli, 2007). Although higher TEER and, or lower P_e values were described for some bovine and porcine models (Zenker et al., 2003; Hoheisel et al., 1998), they have not been extensively

characterized for drug permeability except for the bovine brain endothelial cell and rat glia co-culture model (Cecchelli et al., 2007).

Brain endothelial cells in the triple co-culture EPA model, which shows the tightest paracellular barrier for ions and fluorescein among the seven models tested in our experiments, expressed the highest level of TJ proteins occludin, claudin-5 and ZO-1. The localization of claudin-5 and ZO-1 was also more restricted to the interendothelial junctions in the EPA model than in brain endothelial cells cultured alone. The higher expression and junctional localization of claudin-5 is especially important in the new model, because this TJ protein is the only one so far which has been directly linked to restricted BBB permeability *in vivo* (Nitta et al., 2003). Angiopoietin-1 is a likely candidate molecule to mediate this effect, because it is upregulated in both pericytes and astrocytes by glucocorticoid treatment (Kim et al., 2008) and pericyte-derived angiopoietin-1 enhanced occludin gene expression in a brain endothelial cell line (Hori et al., 2004).

In addition to the induction of barrier properties the presence of the influx transporter proteins Glut-1 and the efflux transporters Mrp-1 and Pgp was demonstrated in the new model. These transporters are crucial for the nutrient transport and for the extrusion of drugs at the BBB (Pardridge, 2002; Abbott et al., 2006; Zlokovic, 2008). The polarity of the Pgp efflux pump activity was also demonstrated in a functional assay in the triple model. Astrocytes, pericytes and hydrocortisone may all contribute to the expression of BBB transporters in brain endothelial cells. It has been demonstrated earlier, that cerebral endothelial cells when cultured alone lose the expression of efflux transport proteins and this can be reversed by co-culture with astrocytes (Berezowski et al., 2004). In the same study the mRNA expression of MRP6 efflux pump was up-regulated in bovine brain endothelial cells by pericytes (Berezowski et al., 2004). Rat pericytes also enhanced P-glycoprotein activity in a mouse brain endothelial cell line, MBEC4 (Dohgu et al., 2005). Astrocytes, hydrocortisone and cyclic AMP increased the transcript level of Glut-1, Mdr1a (Pgp), Mrp3, Mrp4 and Bcrp in rat brain endothelial cells (Perrière et al., 2007).

4.4. Drug permeability test on the new BBB model

The pharmaceutical industry needs reliable *in vitro* BBB models for predicting BBB permeability of CNS drugs. Any *in vitro* model to serve as a permeability screen should display a restrictive paracellular pathway, a physiologically realistic cell architecture and functional expression of transporter mechanisms (Gumbleton and Audus, 2001). As we could

demonstrate, the new model possesses all these criteria. A further advantage of the rat BBB model is that it can be easily compared to *in vivo* results and measurements, and it is simple to construct syngenic cultures. We tested the drug permeability of the new model with a set of 19 compounds, the largest set ever tested on primary cell-based *in vitro* rat BBB model. From the results it was possible to discriminate between passively and actively distributed drugs, compounds with good penetration to the CNS and ligands of efflux transporters. Moreover, a good correlation, $R^2 = 0.89$, was obtained when data from the *in vitro* model were compared with *in vivo* data.

The only rat BBB model which has been tested for 22 compounds in a comparative study, is an SV40-immortalized rat brain endothelial cell line called SV-ARBEC (Garberg et al., 2005). The TEER values of monolayers were only $50\text{-}70 \Omega \times \text{cm}^2$, correlation between *in vitro* and *in vivo* permeability coefficients was low and the model could not distinguish between passively and actively distributed compounds. Our results compare favorably to the best BBB model tested in that study, bovine brain endothelial cells cultured with rat glia cell, which also could discriminate between passively transported compounds and substrates of active efflux (Garberg et al., 2005) and gave high correlation between *in vivo* and *in vitro* permeability data in earlier reports (Cecchelli et al., 1999).

4.5. Conclusion

In vitro reconstituted BBB models are important research implements to study the structural and functional organization of the BBB under physiological and pathological conditions. Furthermore pharmacological studies on reliable and reproducible *in vitro* BBB models can accelerate the research and development of new drugs with better brain penetration. The syngenic rat BBB model established and characterized in this study could be a new tool for basic research and pharmaceutical screening.

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Legend to Figures

Fig 1 Schematic drawing of the preparation of the *in vitro* BBB model. Rat brain endothelial cells (RBEC) are isolated 4 days before the establishment of the co-culture system. To purify cultures cells are kept in the presence of puromycin for two days. Rat astrocytes are seeded at the bottom of 12-well plates, while rat brain pericytes at the filter membranes of inverted cell culture inserts the day before the start of the co-culture. On day 0 RBECs are added to the luminal compartment of the inserts having pericytes on the other side and positioned in the 12-well plates containing the astrocytes. From day 1 cells are grown in culture medium containing 500 nM hydrocortisone. Experiments were performed on day 4.

Fig. 2 Characterisation of primary cultures by immunofluorescence microscopy. Brain endothelial cells express factor VIII-related antigen/von Willebrand factor, while astrocytes are positive for glial fibrillary acidic protein (GFAP). Pericytes give a positive immunostaining for α -smooth muscle actin and NG2, markers of brain pericytes while are negative for endothelial or glial markers. Bar=50 μ m.

Fig. 3 Transmission electron microscopy images of brain endothelial cells (A, B, C) and brain pericytes (D, E) grown in the triple co-culture model on the two sides of the filter membrane of Transwell inserts, marked on pictures as membrane. Brain endothelial cells show typical morphology with oval nucleus (A), many mitochondria (A, B) and tight intercellular junctions (C). There are no junctions between pericytes which have more round cell bodies and nuclei (D) but also long thin processes (E). Bar=600 nm.

Fig. 4 Transendothelial electrical resistance (TEER, expressed as $\Omega \times \text{cm}^2$) and endothelial permeability coefficient for sodium fluorescein (Na-F P_e , expressed in 10^{-6} cm/s) of the different blood-brain barrier models constructed from brain endothelial cells (E), astrocytes (A) and pericytes (P). All data are presented as means \pm S.E.M. (n=8). Statistically significant differences ($P < 0.05$) in TEER are indicated compared to E00 (a), EA0 (b), E0A (c), EP0 (d), E0P (e) and EAP (f), whereas these in P_e are indicated compared to E00 (A), E0A (B), respectively.

Fig. 5 Expression of the tight junction integral membrane proteins occludin and claudin-5, and the cytoplasmic tight junction protein zonula occludens-1 (ZO-1) in different blood-brain

barrier models detected by Western blot. The relative level of the proteins was determined by densitometry. Brain endothelial cells were kept in co-culture with astrocytes in contact (EA0) or out of contact (E0A), with pericytes in contact (EP0) or out of contact (EPA), or with both pericytes and astrocytes (EAP, EPA).

Fig. 6 Immunofluorescent staining of confluent brain endothelial cell monolayers alone (E00) and in triple co-culture (EPA) for tight junction proteins ZO-1 and claudin-5. Arrows show zipper-like staining between endothelial cells in the E00 model, while the junctional immunostaining of endothelial cells in co-culture (EPA) forms a continuous, smooth, pericellular, belt-like pattern (arrowheads). Bar=20 μ m.

Fig. 7 Expression of the influx transporter protein glucose transporter-1 (GLUT1) and the efflux transporters multidrug resistance protein-1 (Mrp1) and P-glycoprotein (Pgp) in brain endothelial cells cultured in the presence of brain pericytes and astrocytes (EPA model) by Western blot and immunohistochemistry. The function of Pgp was tested by the transport of its ligand rhodamine 123 (R123) in the apical to basolateral (A to B) and basolateral to apical (B to A) directions.

Fig. 8 Endothelial permeability coefficients of drugs tested at the *in vitro* blood-brain barrier model (*in vitro* P_e) consisting of brain endothelial cell monolayers cultured in the presence of brain pericytes and astrocytes. Drugs with known penetration to the central nervous system (CNS +) and with low permeability across the BBB and no central effects (CNS -) were selected.

Fig. 9 Correlation between endothelial permeability coefficients of drugs tested at the *in vitro* blood-brain barrier model (brain endothelial cell monolayers cultured in the presence of brain pericytes and astrocytes, EPA model) (*in vitro* P_e) and the apparent permeability coefficients of the same drugs measured in animal models (*in vivo* P_{app}).

Table 1 List of the drugs selected for transport study across brain endothelial cell monolayers cultured in the presence of brain pericytes and astrocytes (EPA model). Drugs with known penetration to the central nervous system (CNS +) are mostly lipophilic and cross brain endothelial cell membranes by lipid-mediated free diffusion. Drugs with low permeability

across the BBB and no central effects (CNS -) are actively extruded by brain endothelial efflux transporters. BCRP, brain cancer resistance protein (ABCG2); MRP-1, multidrug resistance protein-1 (ABCC1); MRP-2, multidrug resistance protein-2 (ABCC2); OATP2, organic anion transporting polypeptide-2 (SLCO1B1); OCT, organic cation transporter (SLC22); Pgp, P-glycoprotein (ABCB1); P_e , endothelial permeability coefficient.

Table 1

Name	MW	CNS	Transport	P_e (10^{-6} cm/s)
antipyrin	188	+	passive lipophilic	222.51
atenolol	226	-	passive hydrophilic	2.49
caffeine	212	+	passive lipophilic	496.67
carbamazepine	236	+	passive lipophilic	198.53
cimetidine	252	-	efflux: Pgp, BCRP, OCT1-3, OCTN-1	2.99
digoxin	781	-	efflux: Pgp, Oatp2	0.44
epinastine	286	-	efflux: Pgp	1.87
hydrocortisone	362	-	efflux: Pgp	5.67
hydroxyzine	448	+	passive lipophilic	1174.04
phenytoin	252	+	passive lipophilic	326.73
prazosin	420	-	efflux: BCRP, Pgp	22.91
propranolole	296	+	passive lipophilic	1987.16
quinidine	783	-	efflux: Pgp, OCT-1	7.54
sulpiride	341	-	efflux: Pgp, influx: OCTN-1, -2, PEPT1	4.39
trazodone	408	+	passive lipophilic	333.61
verapamil	491	-	efflux: Pgp, OCT1-3, OCTN-1	23.51
vinblastine	909	-	efflux: Pgp, MRP-1, -2	3.20
vincristine	923	-	efflux: Pgp, MRP-1, -2	6.32
zolpidem	382	+	passive lipophilic	147.36

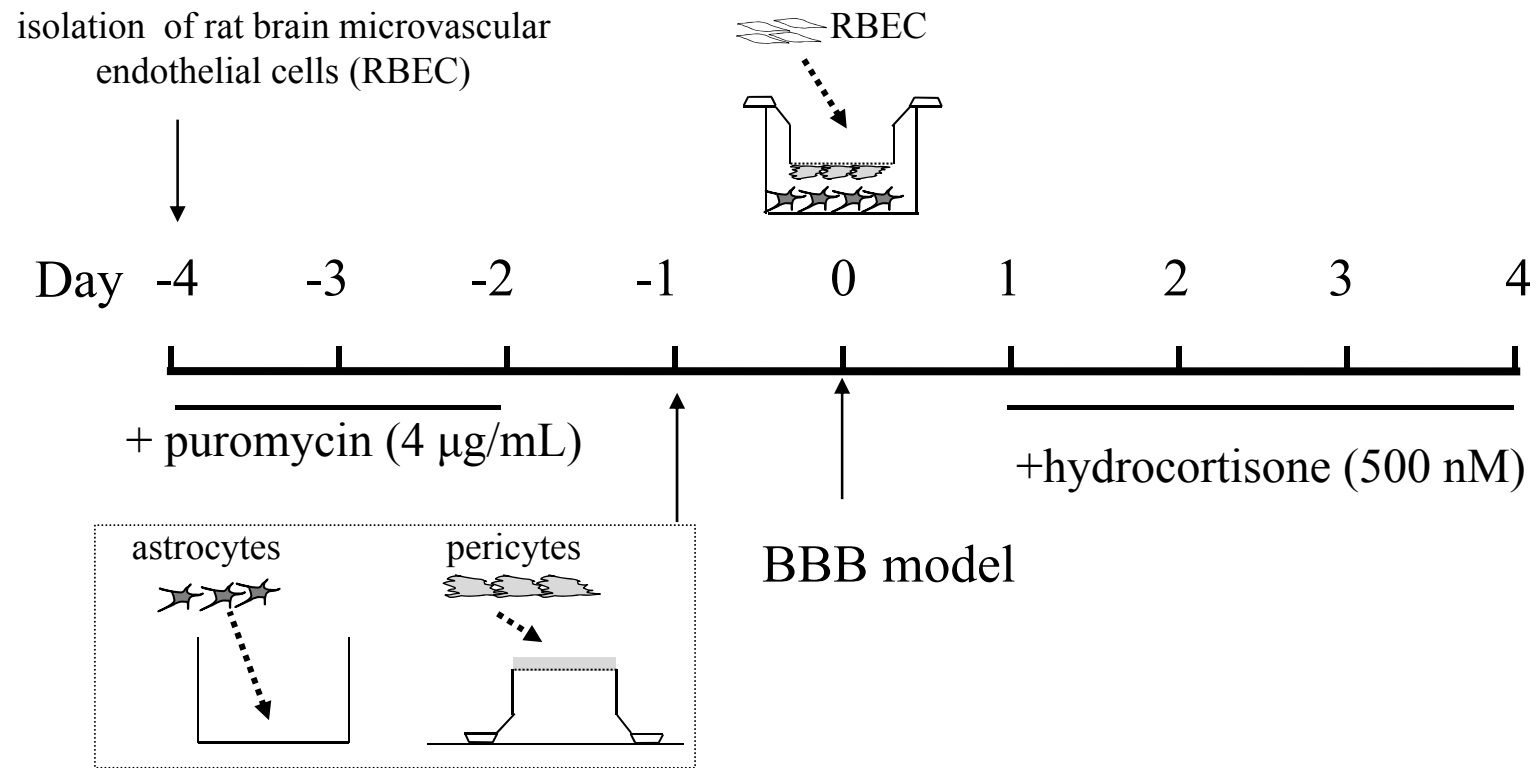


Fig.1

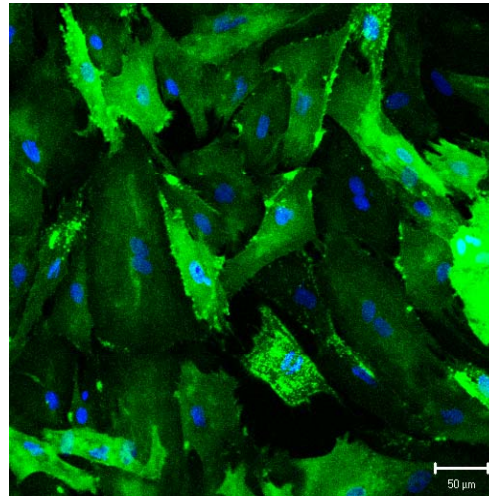
Brain endothelial cells

Pericytes

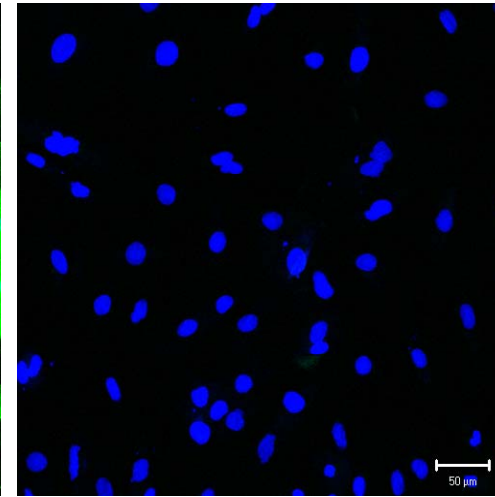
vWF

Astrocytes

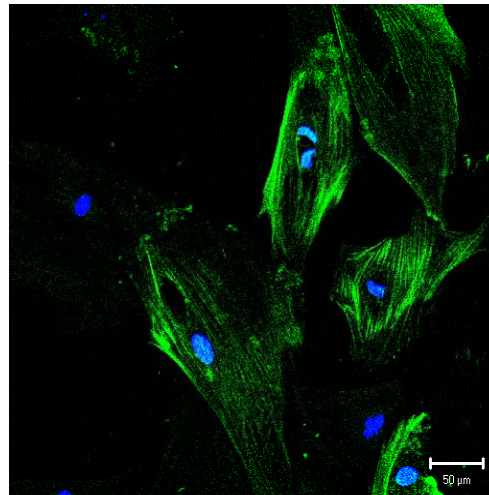
GFAP



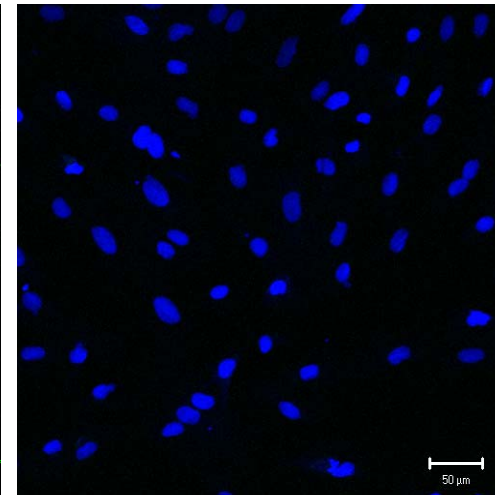
NG2



vWF



α -SM actin



GFAP

Fig.2

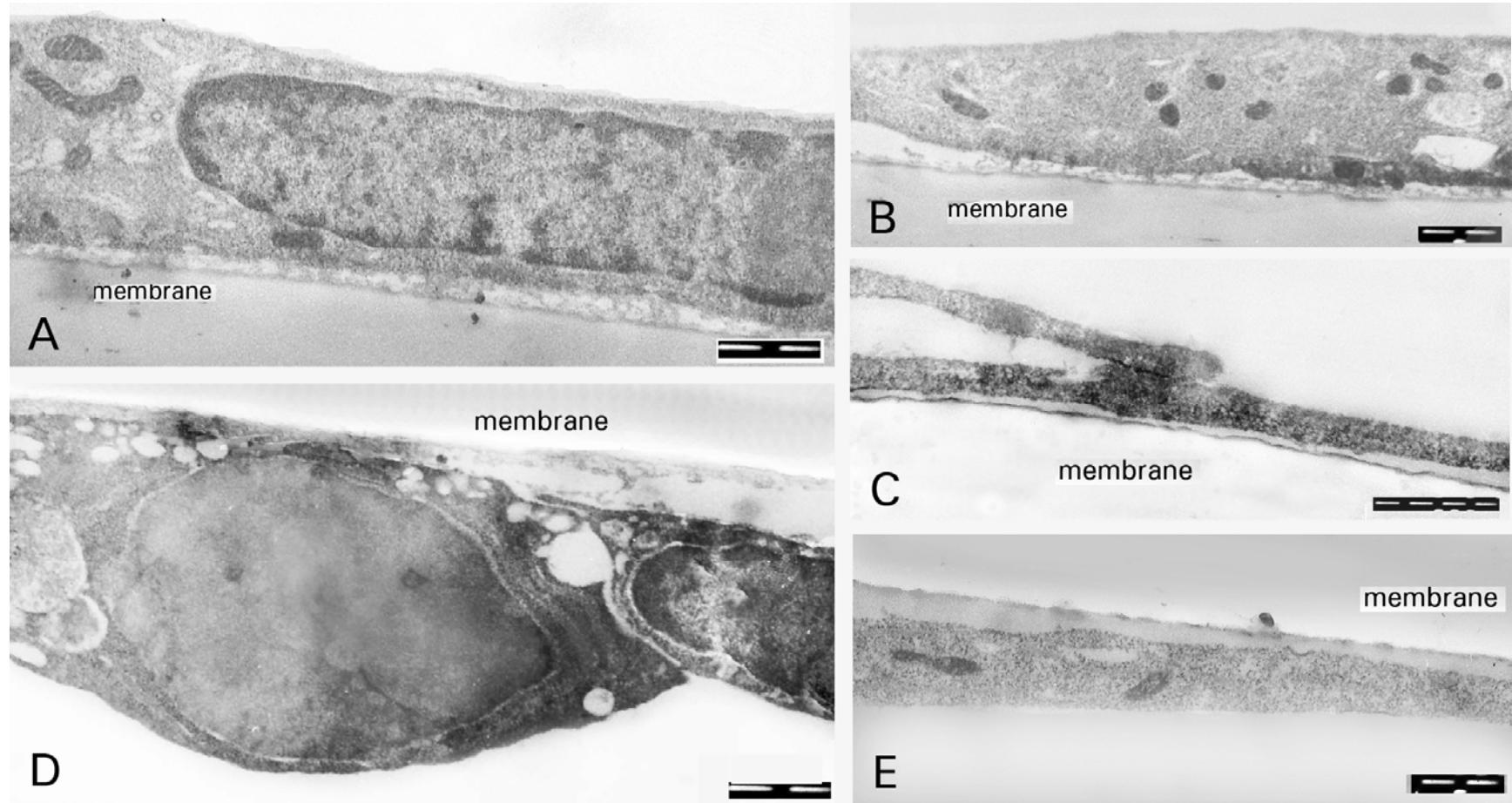


Fig.3

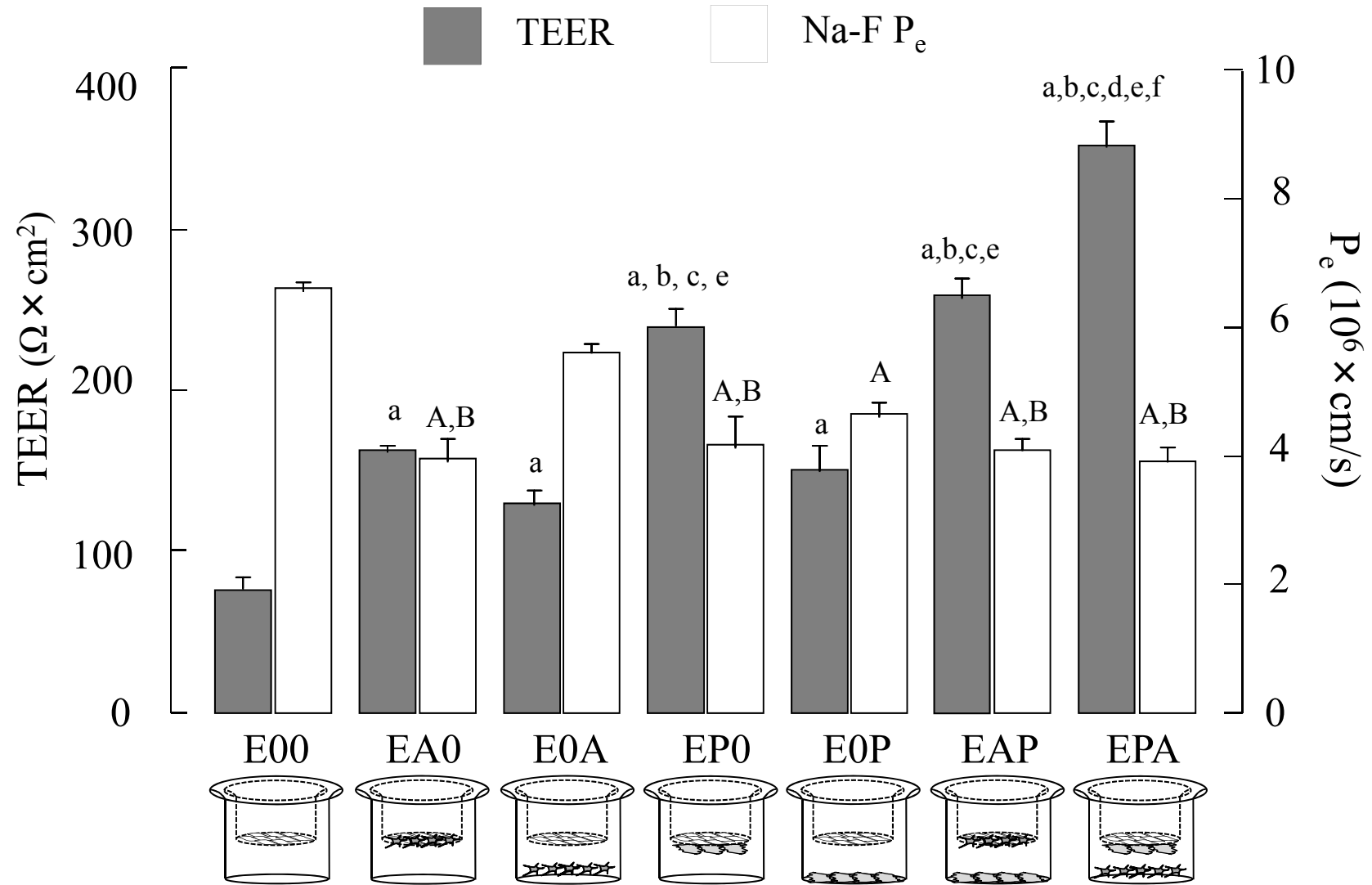


Fig.4

Tight junction protein expression

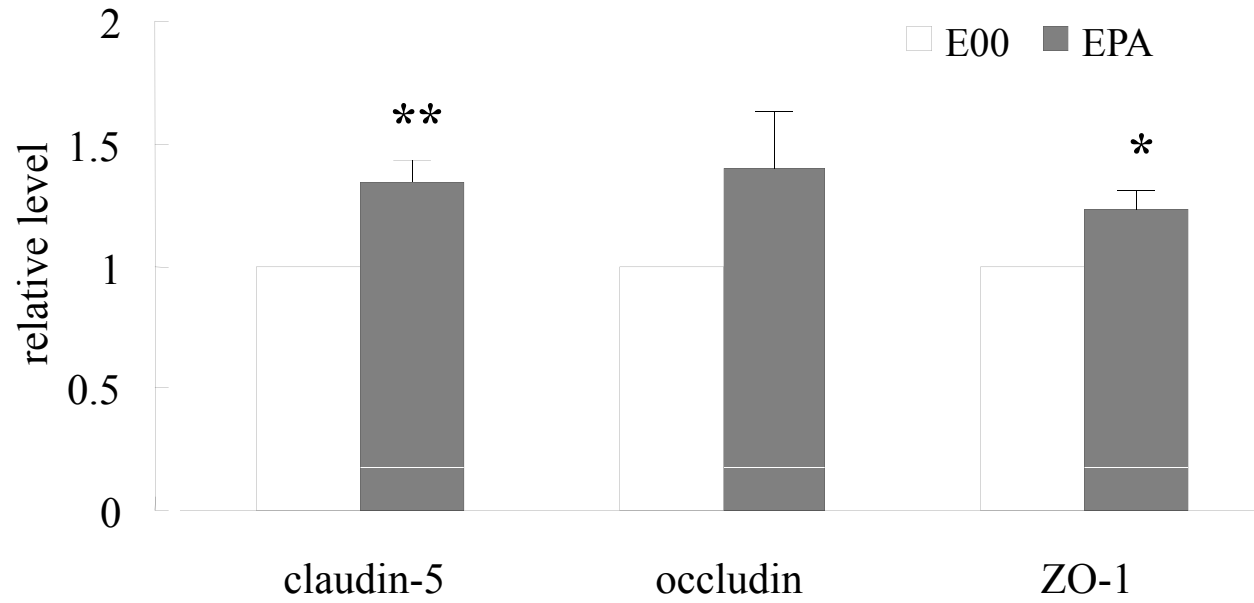
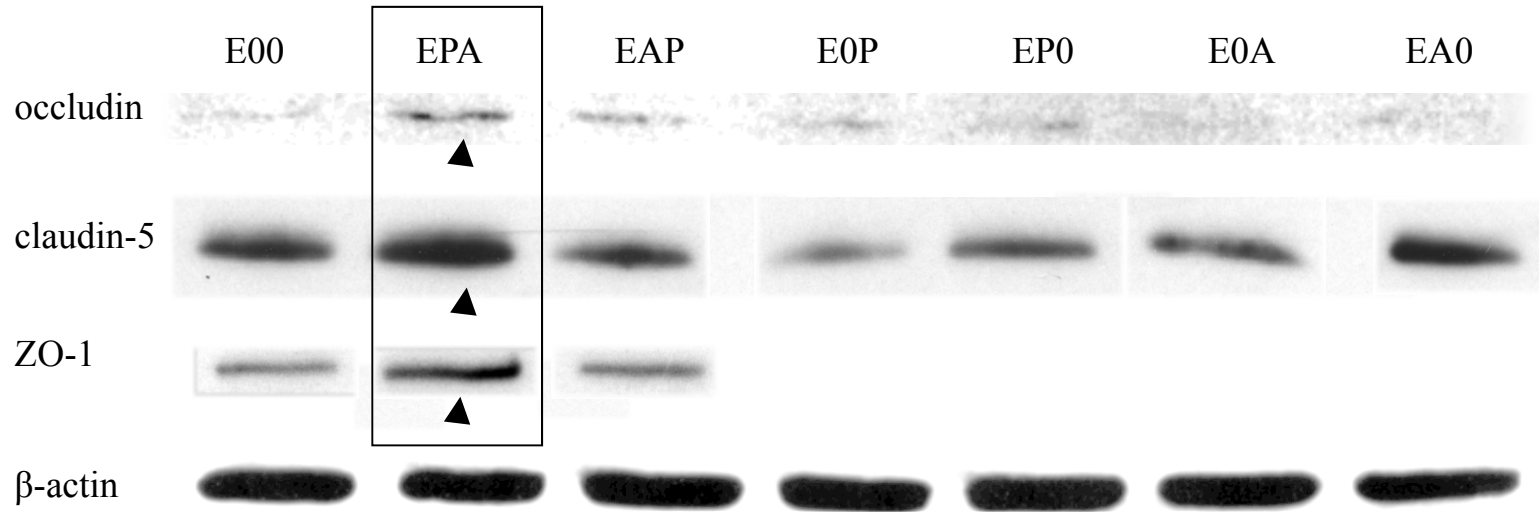


Fig.5

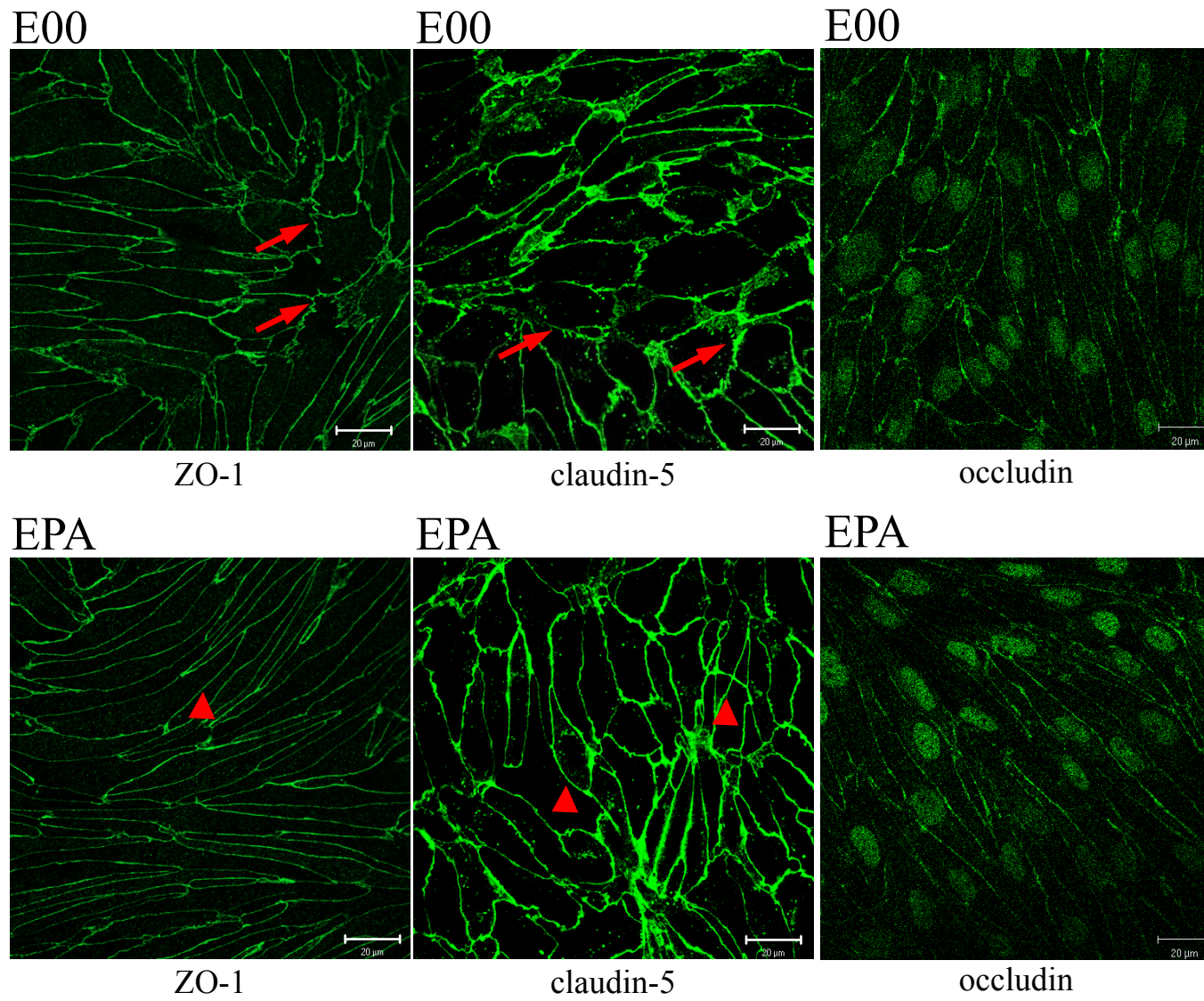


Fig.6

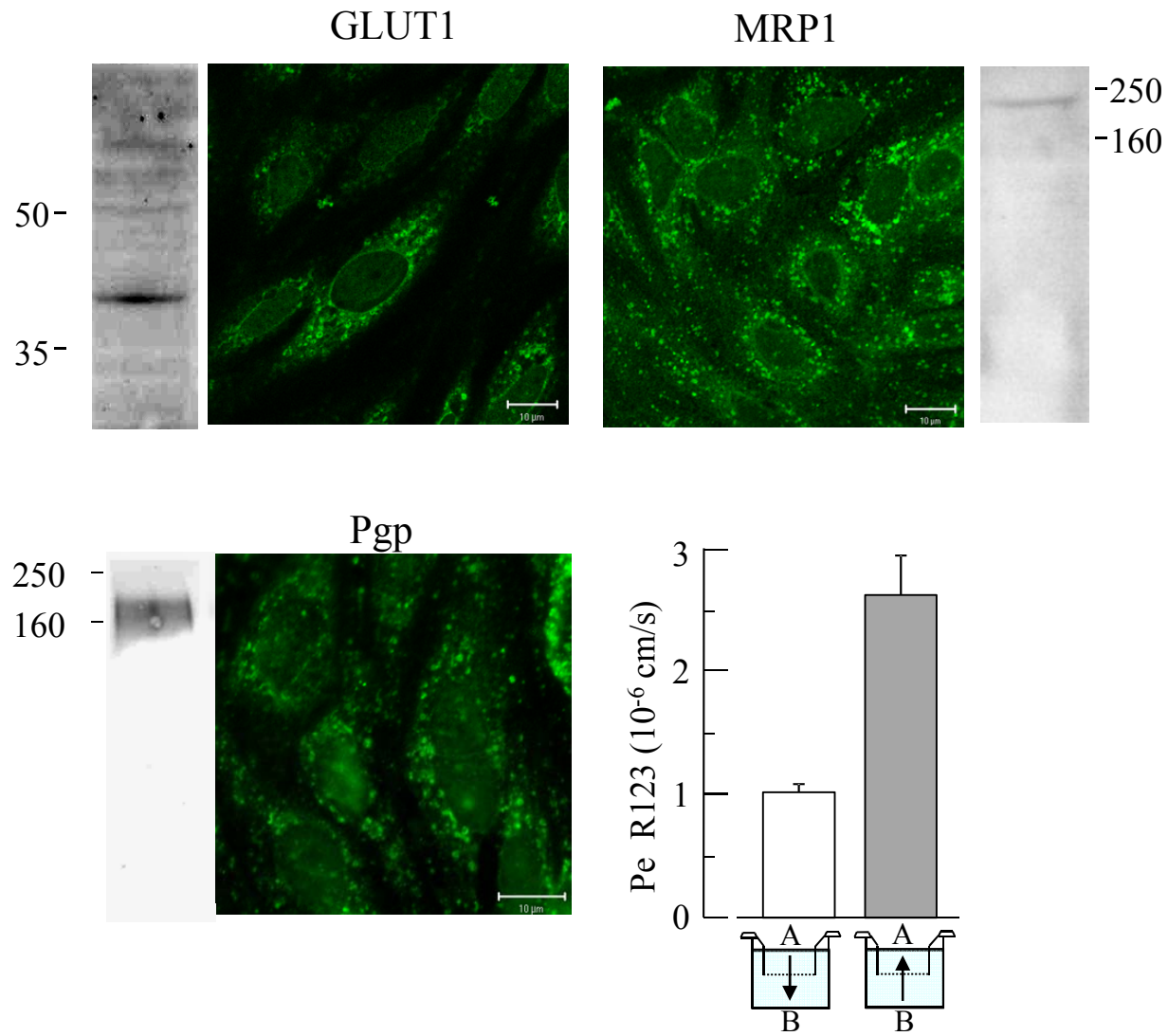


Fig.7

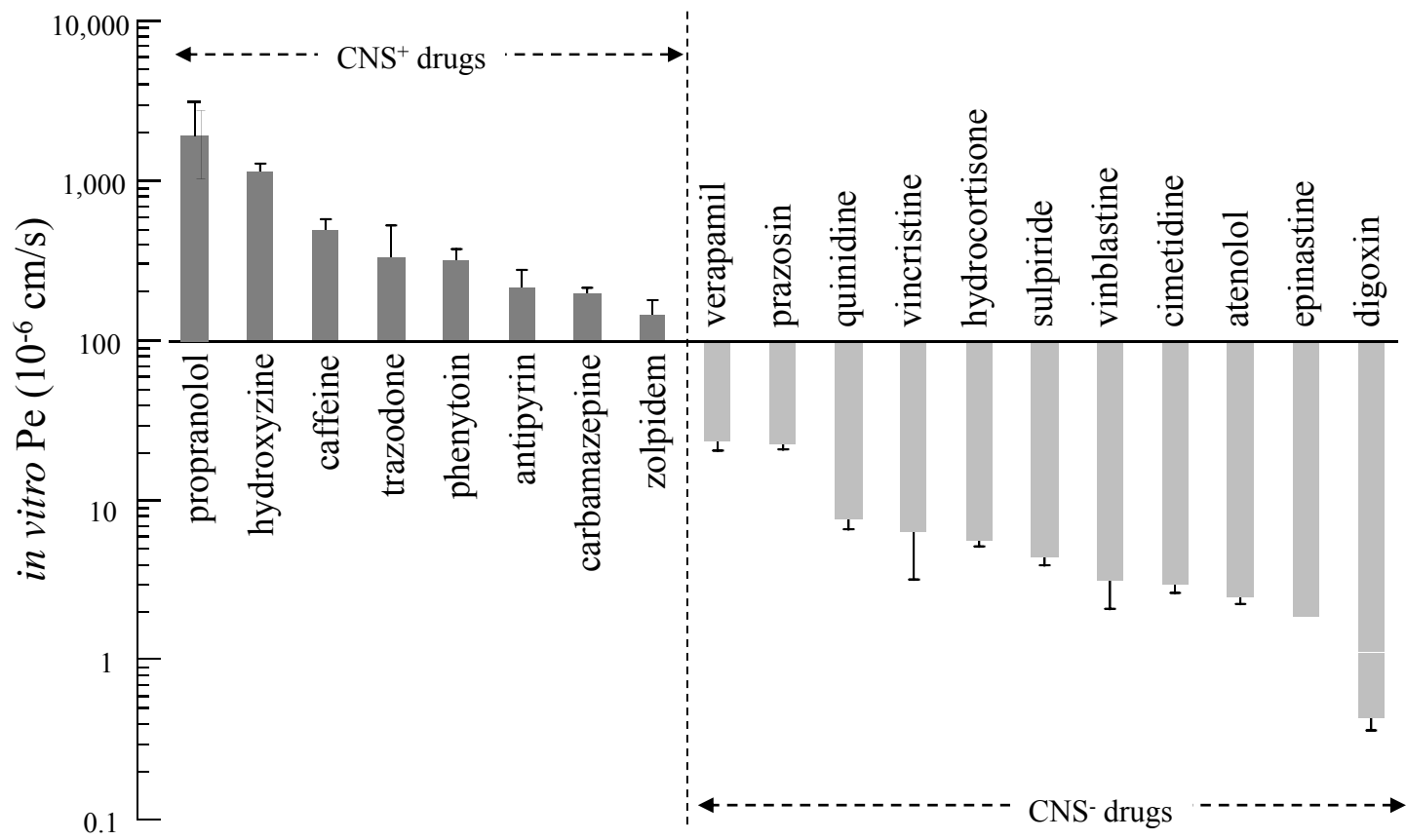


Fig.8

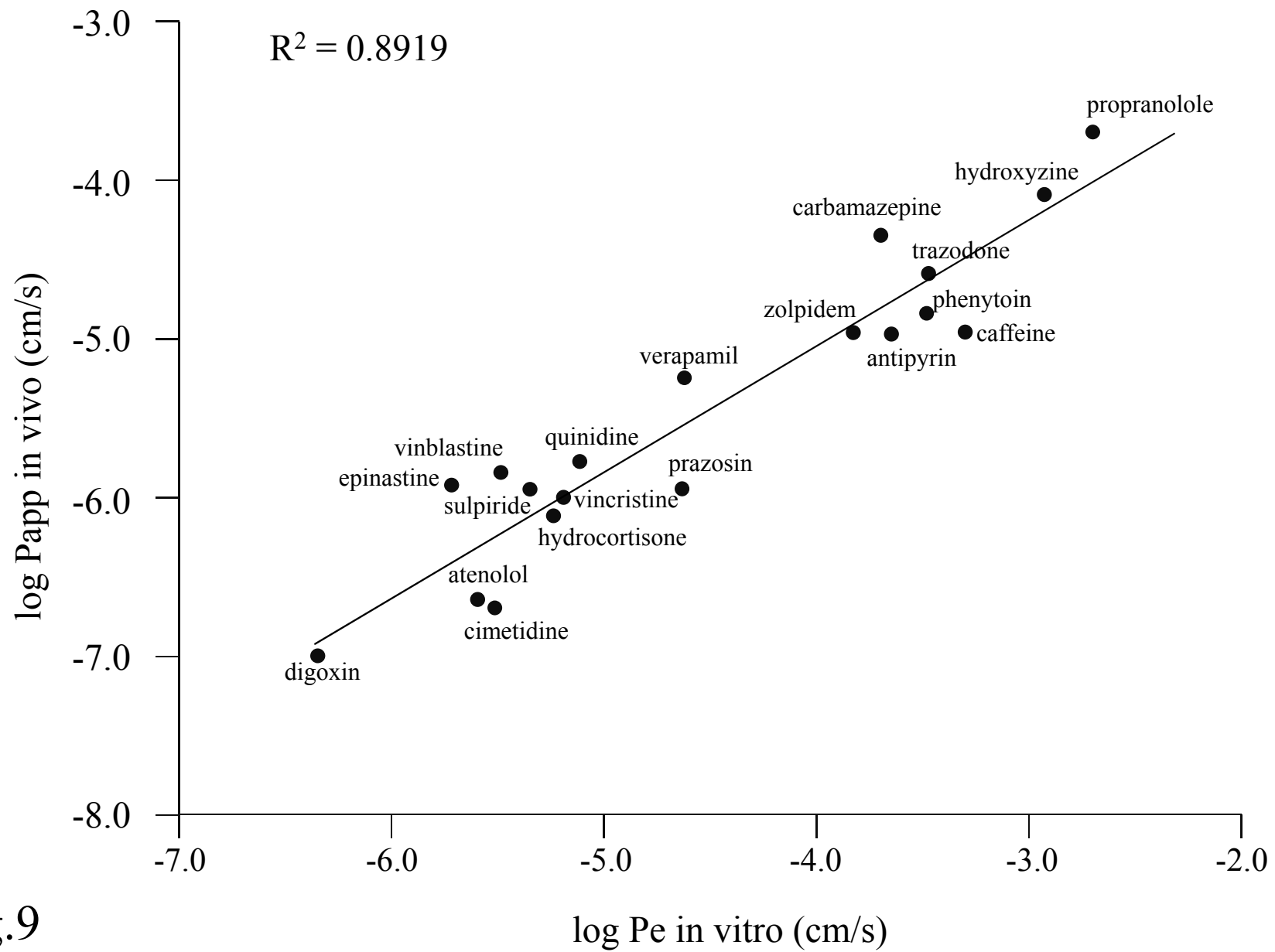


Fig.9